The *Stat3/5* Locus Encodes Novel Endoplasmic Reticulum and Helicase-like Proteins That Are Preferentially Expressed in Normal and Neoplastic Mammary Tissue

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The signal transducers and activators of transcription (STAT) 5 and 3 are critical for mammary alveolar development during pregnancy and remodeling during involution. In the mouse, STAT3, STAT5a, and STAT5b are encoded by adjacent genes on chromosome 11 (60.5 cM). To identify additional genes in the *Stat3/5* locus that may participate in normal and neoplastic development of the mammary gland, we have cloned and sequenced 500 kb and searched for genes preferentially expressed in mammary tissue. We identified six known genes and cloned two new genes, termed *D11Lgp1* and *D11Lgp2*. Both genes are most highly expressed in normal mammary tissue and mammary tumors from several transgenic mouse models. LGP1 consists of 532 and 530 amino acids in mouse and human, respectively (88% similarity). A region in the carboxy-terminal half of LGP1 has limited homology with *Arabidopsis thaliana* GH3-like proteins. Immunofluorescence studies demonstrated that LGP1 is located in the nuclear envelope and the endoplasmic reticulum. LGP2 is a cytoplasmic protein of 678 amino acids.

INTRODUCTION

With each pregnancy, mammary tissue undergoes a cycle of proliferation, differentiation, and regression [1]. These events are controlled by cytokines and their downstream transcription factors. Stimulation of the prolactin receptor in mammary epithelium during pregnancy results in the activation of the signal transducer and activator of transcription-5 gene (*Stat5*), which in turn leads to the proliferation and differentiation of alveolar epithelium [2,3]. Upon weaning, mammary epithelial cells undergo apoptosis followed by tissue remodeling. Activated *Stat3* seems to control mammary epithelial cell death [4], although the inducing signals remain elusive. In the mouse, *Stat3*, *Stat5a*, and *Stat5b* are next to one another on chromosome 11 at 60.5 cM [5]. Expression of *Stat5a* and *Stat5b* is highest in mammary tissue, which correlates with the defects seen in *Stat5a*-null mice [3].

Mice have been generated in which *Stat5a* [3] and *Stat5b* [6], alone or in combination [7], have been inactivated. Because *Stat5a/b*-null mice are infertile and have developmental problems that interfere with mammary studies, it will be necessary to inactivate this locus specifically in mammary epithelium using tissue-specific recombination. In addition, it is necessary to identify genes in this locus that may contribute to alveolar development and tumorigenesis, and whose expression may be altered through the deletion of the locus. To address these issues, we cloned and sequenced 500 kb from the *Stat3/5* locus [5] and searched for genes expressed in mammary tissue and tumors. We identified six known genes. The gene *Ptrf* (polymerase I transcription releasing factor) is located upstream of *Stat3*. The genes *Hcrt* (hypocretin/orexin), *BEC2* (an ether-a-go-go K (+) channel protein), *Gcn5l2* (a histone acetyltransferase), *Dnajc7* (mouse *DnaJ* homologue), and *Cnp1* (29-, 3′-cyclic-nucleotide 3′-phosphodiesterase) are located downstream of *Stat5b* [5].

To identify new genes, we have used a combination of EST database searches, GENSCAN tools for exon prediction, and cDNA cloning. We cloned two novel genes, *D11Lgp1* (*Lgp1)* and *D11Lp2 (Lgp2*), that were expressed preferentially in mammary tissue and tumors.

0888-7543/01 $35.00

doi:10.1006/geno.2001.6661, available online at http://www.idealibrary.com on IDEAL

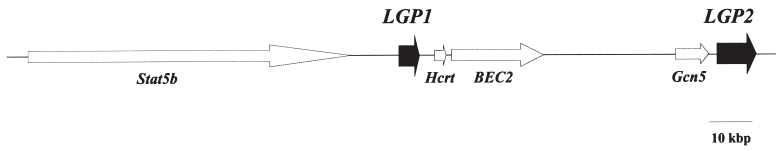

FIG. 1. Structure of the locus containing *Lgp1* and *Lgp2* on mouse chromosome 11. The *Stat5b* part of the BAC clone spans approximately 171 kb and encompasses four known genes and two new genes. *Lgp1* is 4.6 kb and 9.7 kb downstream of *Stat5b*. *Lgp2* spans 9.2 kb and is 76.7 kb downstream of *Stat5b*. The gene sizes and intergenic sequences are drawn to scale.

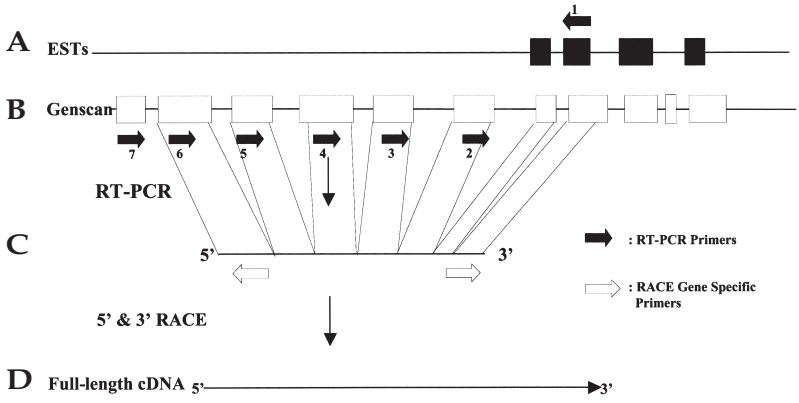

FIG. 2. Strategy for isolating the full-length cDNAs for *Lgp1* and *Lgp2*. (A) Exons were identified by EST search. The black solid line stands for genomic DNA, filled rectangles represent exons found by EST search, and the solid arrow 1 is the downstream RT-PCR primer. (B) GENSCAN software was used to predict additional exons in the genomic sequence. Open rectangles correspond to exons predicted by GENSCAN, and solid arrows 2–7 are the upstream RT-PCR primers. PCR was carried out using different combinations of the primers. (C) The PCR products were sequenced and aligned to the cDNA sequence. 5′- and 3′-RACE techniques were used to obtain the full-length cDNA. Open arrows are RACE gene-specific primers. (D) Full-length cDNA obtained using 5′- and 3′-RACE.

RESULTS

Structure and Genes in the Mouse *Stat5* Locus

To identify genes in the mouse *Stat3/5* locus that are expressed preferentially in mammary tissue and tumors, we cloned and sequenced 500 kb (Fig. 1) and searched the NCBI database using the BLAST algorithm. In addition to *Stat3* and *Stat5a/b*, we identified six known genes and two groups of expressed sequence tags (ESTs), which represented two or more exons of two new genes (*Lgp1* and *Lgp2*). The gene *Lgp1* is 4.6 kb, consists of nine exons, and is located 9 kb downstream of *Stat5b*. *Lgp2* spans 9.2 kb, consists of 12 exons, and is located 76 kb downstream of *Stat5b*. There are three known genes (*Hcrt*, *BEC2*, and *Gcn5l2*) located between *Lgp1* and *Lgp2*.

Cloning and Characterization of *Lgp1* and *Lgp2*

We used one EST from each group to search the NCBI dbEST database for additional ESTs. Four additional ESTs matched with the *Lgp1* sequence and five ESTs matched with the *Lgp2* sequence. The full-length cDNAs encoding *Lgp1* and *Lgp2* were cloned using computational analyses, RT-PCR, and 5′- and 3′-RACE (Fig. 2). The *Lgp1* mRNA was 2.16 kb and contained 92 bp of 5′ UTR, 1596 bp of protein-coding sequence, and 471 bp of 3′ UTR. The *Lgp2* mRNA was 2.4 kb in size, with 2034 bp of protein-coding region and 288 bp of 3′ UTR including a poly(A) tail. We also cloned and sequenced the corresponding human cDNAs, which exhibited a similarity of 80% to those of mouse.

The open reading frames (ORFs) in the mouse and human *LGP1* mRNAs were predicted to encode proteins of 532 and 530 amino acids, respectively. Mouse and human LGP1 exhibited a 73% identity and an 88% similarity. The amino terminus of LGP1 was rich in leucine residues, suggesting this protein might be a secretory or membrane-associated protein. Database analyses demonstrated a limited homology between LGP1 and an auxin-responsive GH3-like protein from *Arabidopsis thaliana* (29% identity and 47% similarity over a stretch of 253 amino acids; Fig. 3).

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human-LGP1        LWPKLQVVVTLDAGGQAEAVAALGALWCQGLAFFSPAYAASGGVLGLNLQPEQP--HGLY 312
mouse-LGP1        LWPKLQVVVTLDSGGQAEAVAALRVLWCQGLAFFSPAYAASGGVVALNLWPERP--QGSY 313
arabi-GH3-like    LFPNAKYVYGIMTGSMEPYVPKLR-HYAGDLPLVSHDYGSSEGWIAANVTPRLSPEEATF 353
                  *:*: : *  : :*.    *. *   :. .*.:.*  *.:* * :. *: *. .  .. :

human-LGP1        LLPPGAPFIELLPVKEGTQEEAASTLLLAEAQQGKEYELVLTDRASLTRCRLGDVVRVVG 372
mouse-LGP1        LLPPGVPFIELLPIKEGTQEEAASTLLLTDAQREKEYELVLTNHTSLTRCRLGDVVQVVG 373
arabi-GH3-like    AVIPNLGYFEFLPVSETGEGEEK-PVGLTQVKIGEEYEVVITNYAGLYRYRLGDVVKVIG 412
                   : *.  ::*:**:.*  : *   .: *:::.:  :***:*:*: :.* * ******:*:*

human-LGP1        AYNQCPVVRFICRLDQTLSVRGEDIGEDLFSEALGRAVGQWAGAKLLDHGCVESSILDSS 432
mouse-LGP1        TYNQCPVVRFTCRLGQTLNVRGEVTDETVFSVALAQAVGQWPGAKLLDHVCVESRVLDSC 433
arabi-GH3-like    FYNNTPQLKFICRRNLILSINIDKNTERDLQLSVESAAKRLSEEKIEVIDFSSYIDVSTD 472
                   **: * ::* ** .  *.:. :   *  :. ::  *. : .  *:      .   :.:

human-LGP1        AGSAPHYEVFVALRGLR--NLSEENRDKLDHCLQEASPRYKSLRFWGSVGPARVHLVGQG 490
mouse-LGP1        EGSAPHYEVFVELRGLR--NLSEENRDKLDNCLQEASAQYKSLRFRGSVGPAKVHLVRPG 491
arabi-GH3-like    PG---HYAIFWEISGETNEDVLQDCCNCLDRAFIDAG--YVSSRKCKTIGALELRVVAKG 527
                   *   ** :*  : *    :: ::  : **..: :*.  * * *   ::*. .:::*  *

human-LGP1        AFRALRAALAACPSSPF-PPAMPRVLR--HRHLAQCLQERVVS------- 530
mouse-LGP1        SFRVLREALAAFSSSSCRPPEMPRVIR--LRHLAQLLQKRVIS------- 532
arabi-GH3-like    TFRKIQEHFLGLGSSAG-QFKMPRCVKPSNAKVLQILCENVVSSYFSTAF 576
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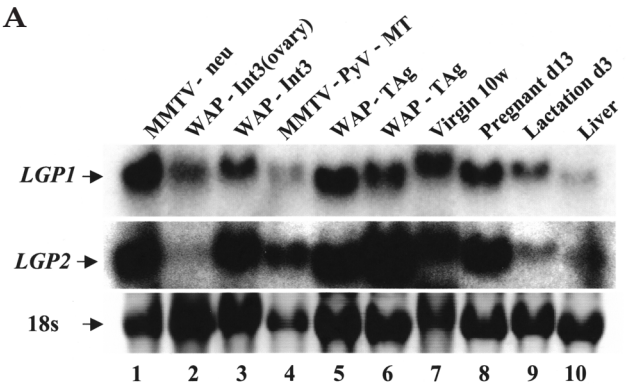
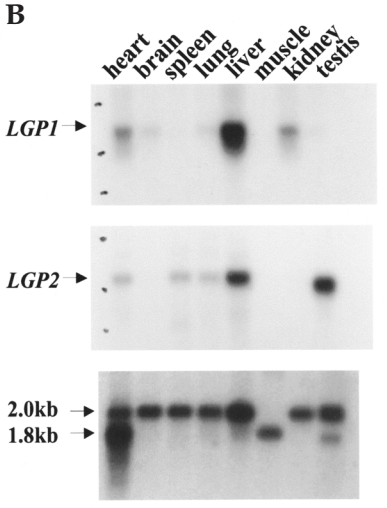
FIG. 3. Alignment of similar region among mouse and human LGP1, and an *A. thaliana* GH3-like protein (GenBank acc. no. AF279129) by CLUSTAL W (1.81) software. We found 29% of the amino acid residues in this region to be identical, and 47% to be conserved.





C

Mouse EST search		
Tissue	*LGP1*	*LGP2*
Mammary gland tumor	4	6
Mammary gland	0	3
Liver	3	1
Kidney	4	0
Spleen	0	3
Testis	0	4
Embryo	6	5
Skin	1	0

*1,955,169 mouse EST sequences were searched.

FIG. 4. Expression of *Lgp1* and *Lgp2*. (A) Expression of *Lgp1* in mammary tissues and tumors. We analyzed 40 μg of total RNA from five mouse mammary tumors (lane 1, 3–6), one mouse ovary tumor (lane 2), three normal mouse mammary tissues (lanes 7–9), and mouse liver (lane 10) by northern blot. (B) Tissue distributions of mouse *Lgp1* and *Lgp2* mRNA using northern blot analysis. *Lgp1* and *Lgp2* mRNA levels were determined in heart, brain, spleen lung, liver, muscle, kidney, and testis. β-Actin is shown as a loading control. (C) Mouse *Lgp1* and *Lgp2* cDNA sequences were screened against the mouse EST database using BLASTN 2.1.2 Basic Search (http://www.ncbi.nlm.nih.gov). All ESTs with high scores were listed in the table. *The number of EST sequences in the mouse EST database at the time of running the BLAST search.

The ORFs in the mouse and human *LGP2* mRNAs suggested a protein of 678 amino acid residues in both species. The identity and similarity of amino acid residues between mouse and human was 79% and 94%, respectively. We identified two conserved domains, DEAD/H and a helicase carboxy-terminal domain. The DEAD/H box domain (amino acids 2–170) in LGP2 showed a similarity of 49% with the DEAD-like helicases superfamily. A 70% similarity (amino acids 415–475) was seen with the helicase superfamily C-terminal domain. Further, there was a conserved ATP binding motif (PTGAGKT), an ATPase motif (DECH), and an unwinding motif (TAS) located within the DEAD/H domain. An RNA binding motif (QARGRARA) was found within the helicase C-terminal domain. A human RNA helicase gene, *RAI1* (retinoic acid-induced gene; acc. no. AF038963), showed similarity to mouse and human *LGP2*.

Tissue Distribution of Mouse *Lgp1* and *Lgp2* mRNAs

We identified *Lgp1* and *Lgp2* ESTs in several cDNA libraries of mammary tumors from transgenic mice (Fig. 4C). The expression of *Lgp1* and *Lgp2* in mammary tissue during normal development and in several mouse mammary models was determined by northern blot analysis (Fig. 4A). High levels of *Lgp1* mRNA were found in mammary tissue from mature virgins and at day 13 of pregnancy, and lower levels during lactation (Fig. 4A). The pattern of *Lgp2* expression was different: mRNA levels were highest during pregnancy and low in the virgin and lactating mammary gland. *Lgp1* and *Lgp2* expression in liver was lower than in mammary tissue. We further analyzed *Lgp1* and *Lgp2* expression in mammary tumors from four different transgenic mouse models and in one ovarian tumor. The *WAP-TAg* [8,9] and *WAP-int3* [10] mice express the SV40-T antigen and the int3/notch4 antigen, respectively, under control of the *WAP* gene promoter. The *MMTV-neu* [11] and *MMTV-PyV-MT* [12] mice express *Erbb2* (avian erythroblastosis oncogene B-2) and the *PyV-MT* (polyoma virus middle T antigen) gene under control of the *MMTV-LTR*. Expression of *Lgp1* and *Lgp2* was detected in all tumors at levels similar to or higher than that in normal mammary tissue. However, their patterns were different (Fig. 4A). Notably, expression of *Lgp2* was lower in the ovarian tumor.

To analyze whether *Lgp1* was expressed in tissues other than the mammary gland, we probed additional northern blots and searched all available EST databases. Membranes containing poly(A)$^+$ RNA from 10 mouse tissues were hybridized with an *Lgp1* cDNA probe, which detected the expected RNA species of 2.2 kb (Fig. 4B). *Lgp1* mRNA levels were highest in liver. Greatly reduced levels were detected in kidney, heart, and brain. The *Lgp2* cDNA hybridized with an RNA of 2.4 kb, and expression was detected in testis, heart, spleen, and lung (Fig. 4B). The results from mouse EST database searches confirmed this tissue distribution. Out of 1,955,169 mouse EST sequences, *Lgp1* sequences were detected in liver, kidney, embryo, skin, and mammary gland tumors (Fig. 4C). *Lgp2* sequences were detected in liver, spleen, testis, embryo, mammary gland tumors, and normal mammary gland tissue (Fig. 4C).

doi:10.1006/geno.2001.6661, available online at http://www.idealibrary.com on IDEAL

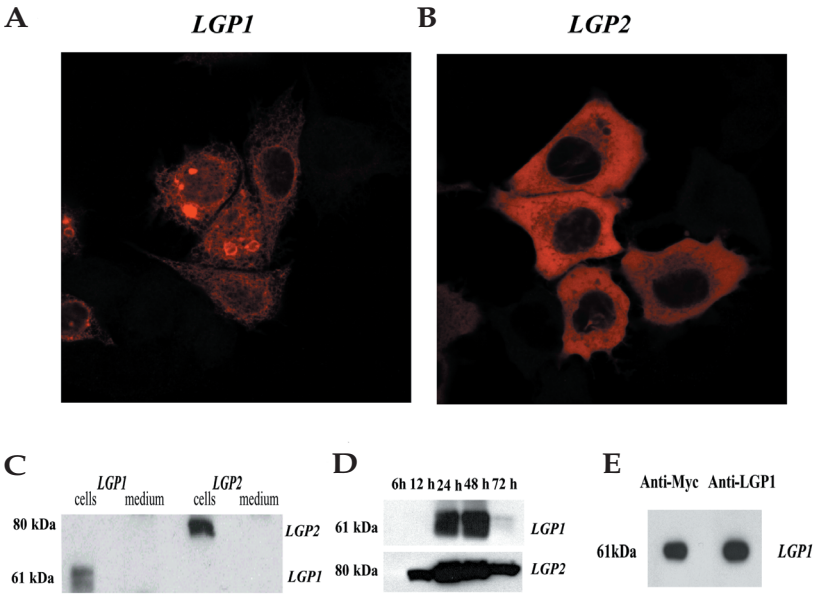


FIG. 5. Localization and analysis of LGP1 and LGP2. (A, B) Localization of mouse LGP1 and LGP2 in HeLa cells using immunofluorescence. HeLa cells were fixed and permeabilized 48 h after transfection, stained with mouse anti-myc antibody, and visualized with Rhodamine-conjugated goat anti-mouse IgG secondary antibody (red). (A) LGP1 is located in the ER. (B) LGP2 is located in the cytoplasm. (C) Analysis of recombinant LGP1 and LGP2. *Lgp1* and *Lgp2* cDNAs were cloned into a mammalian expression vector containing a myc tag. HeLa cells were transiently transfected with the expression vectors. Cell lysates and medium were collected 48 h after transfection, followed by immunoblot analysis to detect the expressions of the recombinant proteins (medium was immunoprecipitated with anti-myc antibody before immunoblotting). (D) Time course analysis of recombinant LGP1 and LGP2 on expression in HeLa cells. Cells were harvested at 6, 12, 24, 48, and 72 h after transfection, followed by immunoblot using anti-myc antibody. (E) Detection of recombinant LGP1 protein by immunoblot using antibody against LGP1.

Localization of Mouse LGP1 and LGP2 within Cells

To identify whether the predicted open reading frames in *Lgp1* and *Lgp2* mRNA encoded proteins and to determine their localization in the cell, we cloned the respective cDNAs into mammalian expression vectors and analyzed the recombinant proteins. A myc tag was fused to the C termini of LGP1 and LGP2, and antibodies against the myc tag were used to identify the two proteins. HeLa cells were transfected with the LGP1 and LGP2 expression vectors and the presence of the respective proteins was analyzed by immunohistochemistry and western blots. Based on confocal microscopy, LGP1 was located in the endoplasmic reticulum (ER) and LGP2 was evenly distributed in the cytoplasm (Figs. 5A and 5B). We also generated antibodies against mouse LGP1, which recognized LGP1 in transfected HeLa cells (Fig. 5E). We detected the endogenous protein in the mammary epithelial cell line HC11 using immunohistochemistry. Although the signal was rather weak, LGP1 was located in the ER (data not shown). Medium and cell lysates were collected 48 hours after transfection and analyzed by immunoblot. The concentrated medium was immunoprecipitated with anti-myc antibodies, followed by immunoblot analysis to identify whether the recombinant proteins were secreted into the medium (Fig. 5C). Recombinant LGP1 and LGP2 of the predicted sizes were detected in cell lysates, but not in the medium. LGP1 appeared as two bands on Tris-glycine gels; the upper band was more pronounced after 48 hours, suggesting that LGP1 may undergo posttranslational modifications. After 48 hours, LGP1 levels had decreased sharply compared with LGP2 at the same time point (Fig. 5D). This suggested that overexpression of LGP1 might be toxic. Alternatively, LGP1 might be less stable than LGP2.

Immunohistochemical analyses demonstrated that LGP1 was located in the nuclear envelope, the ER, and to some extent in circular structures adjacent to the nuclear envelope. To verify that these structures were part of the ER or nuclear envelope, we co-stained with antibodies against the myc tag and calreticulin (a protein of the ER; Fig. 6). The bright circular structures were seen in anti-myc and anti-calreticulin staining at 12 hours and 24 hours after transfection, but very few were seen at 6 hours or later than 48 hours (data not shown). In merged images, the circular structures appeared yellow, suggesting that LGP1 is located in the ER. These experiments also demonstrated that the ER had undergone proliferation, a phenomenon frequently observed in cells where large amounts of protein are expressed, such as HMG-CoA reductase [13]. In our experiment, the proliferation of the ER may have accommodated the overexpression of LGP1.

DISCUSSION

Here we report the identification and characterization of a novel nuclear envelope/endoplasmic reticulum-resident protein (LGP1) and a cytoplasmic protein (LGP2) that are preferentially expressed in normal and neoplastic mammary tissue. The respective genes are located in the *Stat5* locus, which is involved in mammary development.

There are two lines of evidence that suggest LGP1 is an ER resident protein: it possesses a hydrophobic N terminus and immunofluorescence studies demonstrated subcellular localization of myc-tagged LGP1 in the ER. This observation is supported by co-staining for the ER protein calreticulin. We hypothesize that LGP1 is a new type of ER resident protein that lacks homology to any of the known proteins resident in the ER. Based on the hydrophobic N terminus, it is possible that LGP1 is a secreted protein. However, we were unable to detect it in the supernatant of cells. A region in the

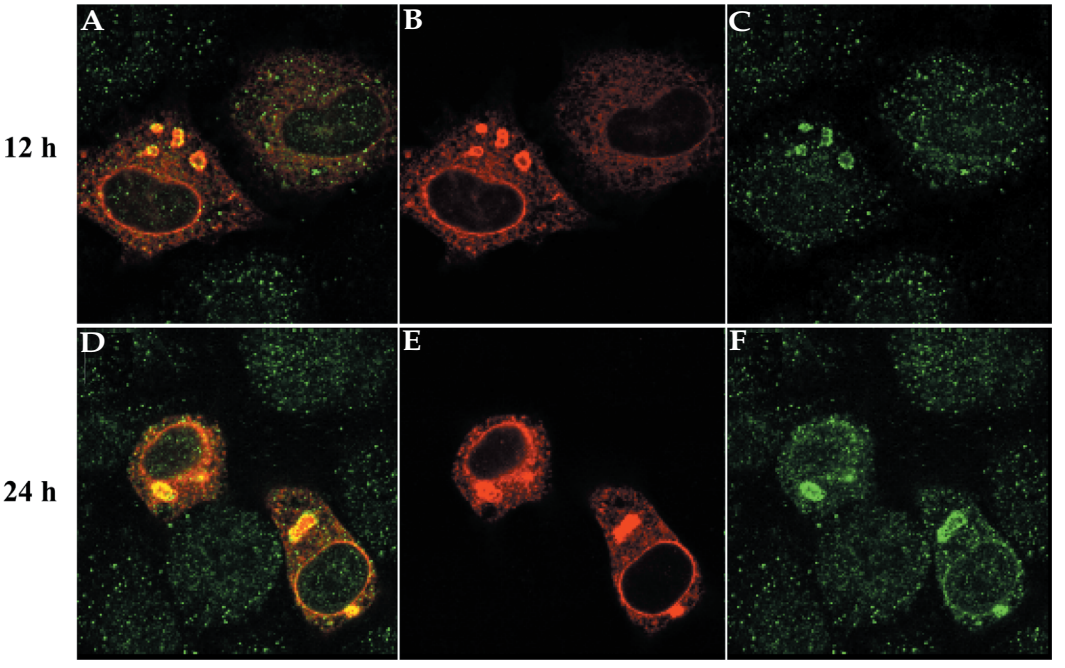


FIG. 6. Co-localization of LGP1 with calreticulin in transfected HeLa cells. At 12 h and 24 h after transfection, immunofluorescence studies were carried out using a mouse anti-myc antibody to probe for LGP1 and visualized with Rhodamine-conjugated goat anti-mouse IgG secondary antibody (B and E, red). Cells were co-stained with rabbit anti-calreticulin and visualized with Alexa 488 goat anti-rabbit secondary antibody (C and F, green). In merged images, yellow indicated colocalization of the two proteins (A and D).

C-terminal part of LGP1 resembles GH3-like proteins. The GH3 protein was first identified in soybeans as an early auxin-responsive gene [14]. Auxins are a class of plant hormones that affect a wide range of growth and developmental processes [15]. GH3 is one of the most specifically auxin-regulated genes [16]. In *A. thaliana*, the family of GH3-like proteins shares 25–29% identity with LGP1 over a stretch 280 amino acids. The functions of GH3 proteins are not known, and no mammalian homologues have been described.

LGP2 is a cytoplasmic protein that shares similarities with members of the DEAD/H box family of proteins. Two conserved regions, the DEAD and helicase C-terminal domain, were identified in LGP2. RNA helicases exhibit several functions including translational initiation, ribosome biogenesis, nuclear mRNA export, RNA degradation, and nuclear as well as mitochondrial RNA processing. Other proteins of this family have been implicated in cell growth, division, and differentiation [17]. Therefore, RNA helicases represent key elements in the regulation of different cellular processes. Except for the conserved functional domains, sequences vary greatly between the RNA helicases. The biological function of LGP2 is unknown.

Expression of *Lgp1* and *Lgp2* was highest in normal mammary tissue and mammary tumors from several transgenic mouse models. *Lgp1* mRNA levels were similar in mammary tissue from virgin and pregnant mice, suggesting that the gene is not under the control of lactogenic hormones. Together with the reduced expression during lactation, this suggests that *Lgp1* is expressed in the stromal and epithelial compartments. High levels of *Lgp1* mRNA were detected in mammary tumors from four different transgenic mouse models, suggesting that it serves as a marker for transformed mammary epithelium. The pattern of *Lgp2* expression was different from that of *Lgp1* with maximal mRNA levels during pregnancy and low in the virgin and lactating state. Such expression profiles are different from those observed with genes under lactogenic control and therefore point to potentially new control mechanisms. Although *Lgp2* mRNA was found in every mammary tumor tested, its pattern of expression was distinct from that of *Lgp1*. This suggests that *Lgp1* and *Lgp2* are characteristic of specific cell types or stages of tumor progression. Like *Stat5*, *Lgp1* and *LGP2* are preferentially expressed in mammary tissue. This raises the possibility that the promoters of these genes in the *Stat5* cluster are under coordinate control. Furthermore, it is possible that *Lgp1* has a role in normal [3,18] and neoplastic [19] mammary development.

MATERIALS AND METHODS

Cloning of Lgp1 and Lgp2 cDNA. We cloned and sequenced two BACs from the mouse *Stat3/5* locus that spanned 500 kb in genomic sequence, and searched the NCBI database with this sequence using the BLAST algorithm. Six additional known genes and two groups of expressed sequence tags (ESTs), which represented two or more exons of two new genes (*D11LGP1*, GenBank acc. nos. AF316996 and AF316998; *D11LGP2*, GenBank acc. nos. AF316999 and AF317000), were identified. One EST from each group (*Lgp1*, AW763596 from mammary gland tumor NCI_CGAP_Mam3; *Lgp2*, AW414261 from mammary gland tumor NCI_CGAP_Mam3) were used to search the NCBI dbEST database for additional ESTs. Using the GENSCAN program, the putative coding exons of *Lgp1* and *Lgp2* were predicted. To clone the full-length cDNA, oligonucleotides from the predicted exons were designed, RT-PCR was carried out using total RNA from mouse liver and virgin mammary tissue, followed by

sequence analyses. The RT-PCR fragments were aligned in using the Sequencher 4.0.5 program. The 5′ and 3′ ends of the full-length cDNAs were isolated using the GeneRacer kit (Advanced RACE Method for Amplification of Full-Length cDNA Ends, Invitrogen, L1500-01).

RNA analyses. Mouse multiple tissue northern blots containing 2 μg of poly $(A)^+$ RNA from ten tissues were purchased (Clontech). Mammary tissue northern blots were prepared with 40 μg total RNA from six mouse tumors (*MMTV-neu*, *WAP-Int3*, *MMTV-PyV-MT*, and *WAP-TAg*), normal mouse mammary tissues (virgin 10 weeks, pregnant day 13, lactation day 3), and mouse liver. PCR fragments were used as hybridization probes. The *Lgp1* probe was generated with primers 5′-ATCTTCCACTGACCCAAACC-3′ and 5′-CAGATGGACTTTGGCAGGAC-3′ and spanned 1.2 kb. The *LGP2* probe was generated with primers 5′-AGGTGGTATAGTAGACCGAG-3′ and 'GATGCCTGACTTGAAGCAAC-3′ and spanned 1 kb. The probes for northern blot analysis were labeled with [α-^{32}P], using the Prime-it II random priming kit (Stratagene), and diluted in Express Hybridization Solution (Clontech). Blots were hybridized at 65°C overnight and washed according to the manufacturer's instructions.

Construction of expression vectors. The full-length coding regions of *Lgp1* with *Eco*RI/*Xba*I overhangs and *Lgp2* with *Not*I/*Apa*I overhangs were generated using PCR catalyzed by Platinum *Taq* HIFI (Gibco BRL). The *Lgp1* cDNA was constructed with primers 5′-GGAATTCATGCTTCTGCTGTGGCTGCTG-3′ and 5′-GCTCTAGAGGATATCACCCTCTTCTGCAG-3′. The *Lgp2* cDNA was constructed with primers 5′-AAGGAAAAAAGCGGCCGCATGGAGCTGCGA-3′ and 5′-ACCGGGCCCGTCCAGGGAGAGCTCAGACA-3′. The *Lgp1* and *Lgp2* cDNAs were cloned into pcDNA4/Myc-His mammalian expression vector (Invitrogen) using *Eco*RI/*Xba*I restriction sites for *Lgp1* and *Not*I/*Apa*I for *Lgp2*. *Myc* and *His* epitope tags were fused in-frame at the C terminus of *Lgp1* and *Lgp2*. The integrity of the constructs was confirmed by DNA sequencing.

Transient expression of Lgp1 and Lgp2 in mammalian cells. HeLa cells were grown and maintained in DMEM medium, supplemented with 10% (v/v) fetal bovine serum (Gibco BRL), 100 units/ml penicillin, and 100 mg/ml streptomycin. We seeded 2 to 3×10^5 cells into individual wells of a six-well plate 24 h before the transfection. Plasmid DNA for transfection was prepared using the Qiagen maxi-prep protocol. FuGENE6 transfection Reagent (3 μl; Roche Molecular Biochemicals) and 1 μg plasmid DNA were incubated for 30 min at room temperature in 100 μl serum-free medium. Cells at 60–80% confluency were transfected with the FuGENE6-DNA complexes in 1 ml serum-free medium for 5–6 h at 37°C, and then 1 ml medium with 10% (v/v) fetal bovine serum was added. Transfected cells were cultured at 37°C (5% CO_2). Cells and medium were harvested 6, 12, 24, and 48 h after the start of transfection.

Antibodies. A peptide specific for mouse LGP1 (ELRGLRNLSEENRDKLD) was conjugated to keyhole limpet hemocyanin and used for immunization of rabbits. The specificity of the antiserum was analyzed by western blots using protein extract of HeLa cells transfected with the LGP1 expression vector. Antibody against the myc tag was purchased (Invitrogen).

Immunofluorescence. For immunofluorescence studies, cells were seeded onto sterilized glass coverslips contained in six-well plates. Transfected cells were fixed in 4% formaldehyde (v/v) in phosphate-buffered saline (PBS) for 30 min at room temperature, and permeabilized with 0.1% Triton X-100/PBS for 3 min. Cells were incubated with 1 μg/ml mouse anti-myc antibody (Invitrogen) in blocking buffer (5% goat serum (Gibco BRL), 1% bovine serum albumin (ICN Biomedicals Inc.) in PBS for 1.5 h at room temperature. Then cells were washed three times with blocking buffer, incubated with Rhodamine-conjugated goat anti-mouse IgG in the same buffer for 45 min, and washed three times using PBS. For ER double staining, anti-human calreticulin (10 μg/ml) was used as the primary antibody and Alexa 488 anti-rabbit IgG as the secondary antibody; the procedure was the same as above. After the final wash, each coverslip was prepared for microscopic examination by applying VectaShield mounting medium. Cells were viewed using an Axiovert 100 TV (Zeiss, Germany) inverted microscope. Images were captured with a LSM 410 Lasa-scanning confocal microscope, and analyzed using the LSM software. Adobe Photoshop (Adobe Systems Inc.) was used for final processing of the images.

Immunoprecipitation and immunoblotting. Medium (3 ml) was concentrated by Centricon-10 columns (Amicon) to 500 μl, and 2 μg anti-myc antibody was added and incubated with rotation for 1 h at 4°C. Immune complexes were sedimented with 60 μl immobilized Protein A beads (Sigma) overnight at 4°C. The beads were washed three times in 1× lysis buffer (20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 10% (v/v) glycerol, 1% (v/v) NP-40, 2 mM EDTA, 10 mM NaF, 50 μg/ml PMSF, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM sodium orthovanadate), resuspended in 100 μl 2× loading buffer (250 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.006% bromophenol blue), boiled for 3 min, and centrifuged briefly. Transfected HeLa cells were washed in ice-cold PBS and scraped into 1 ml PBS, centrifuged briefly, and resuspended in 100 μl 2× lysis buffer, rotated for 1 h at 4°C, and centrifuged at 13,000*g* for 10 min at 4°C to remove insoluble debris. Protein (25 μg) from cell lysates and protein (40 μl) from medium were loaded to precasted 8% Tris-Glycine gels, and proteins were transferred to PVDF membranes as instructed by the manufacturer (Novex).

ACKNOWLEDGMENTS

We thank Mei Zhang for advice and technical assistance, and the members of the Laboratory of Genetics and Physiology for advice and critical reading of this manuscript.

RECEIVED FOR PUBLICATION JUNE 20;
ACCEPTED SEPTEMBER 27, 2001.

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